OBSERVATIONS ON FEHLING'S TEST FOR DEXTROSE IN URINE

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Interpretation of Certain Results Obtained with Fehling's Test

For the detection of a comparatively large amount of sugar, such as is generally in evidence in ordinary diabetic urine, Fehling's test gives very definite results. When applied, however, in the usual way its indications are so often ambiguous as to render it of little service for the detection of small amounts of sugar. Its reactions, moreover, are not constant, for an amount of sugar sufficient to give a fairly distinct reduction and precipitation of cuprous oxide or hydrate in a certain urine, may in another sample give little or no indication of its presence on the most careful application of the test as ordinarily applied. On the other hand, such a modified reaction may be obtained as to render the interpretation difficult or impossible, one observer, perhaps, thinking that a trace of sugar is present, another crediting excess of some 'interfering' substances with the production of the change. Again, it would seem that every urine is capable of giving a well-marked reaction with Fehling's solution if equal parts of the solution be mixed with the urine and boiled for some time; the reaction usually consists in the separation of a well-marked precipitate, generally of a yellowish appearance—probably cuprous hydrate, or a mixture of the latter with the red cuprous oxide. The time required for the production of this reaction varies greatly in different urines, but in general, boiling for about three to four minutes is sufficient; sometimes less than two minutes is required, while in other cases up to eight minutes may be necessary. Since Fehling's solution mixed with water gives no yellow or red precipitate

however long it is subjected to heat, it is obvious that the phenomenon is not the result of prolonged boiling on Fehling's solution, but depends directly on the urine. This reduction on prolonged boiling with normal urine suggests the necessity for limiting the amount of boiling when testing for sugar in doubtful cases.[1]

If the application of the test be modified, more accurate results can certainly be obtained with comparatively small amounts of sugar, but this necessitates more time and labour than the majority of clinicians are prepared to give, and at the same time renders the test somewhat complex in application. Of these modified methods that advocated by Allen[2] is perhaps the best; the greater accuracy obtained by this method depends on the utilization of the copper sulphate part of Fehling's solution for the precipitation of the majority of interfering substances present in the urine, the addition of a trace of acetic acid enhancing the result; uric acid, xanthine, hypoxanthine, and albumin (if present) are thrown down *in toto*, but phosphates and kreatinin only partially. After separation of these substances by filtration, the greenish-blue liquid obtained is boiled with the alkaline part of Fehling's solution and reduction takes places more or less quickly in proportion to the relative amounts of dextrose present. In the presence of from 0·2 to 0·3 per cent. of sugar separation of cuprous oxide occurs before the boiling point is reached, but with smaller quantities deposition takes place during the cooling of the solution, which first becomes greenish, ultimately giving a yellow precipitate. In applying the above modification, however, it was found that small amounts of sugar could not always be detected, and that some interfering substances seemed in many cases to be still present. Normal urines to which small amounts of chemically pure dextrose had been added often failed to respond even to Allen's modification, and different urines seemed to react differently, for while it was often easy to detect a certain amount in one urine, it was sometimes impossible to recognise dextrose in another containing

1. A solution of kr.atinin in water containing, approximately, the same percentage of kreatinin as ordinary urine gives, on *prolonged* boiling, quite a similar reduction to that obtained from urine; it would thus seem that the reduction observed on boiling normal urine for some considerable time is due to kreatinin.

2. Allen, *Chemistr of Urine*, 1895, pp. 62-63.

twice the amount of sugar added to the first. In the absence of interfering substances, however, Fehling's solution is one of the most delicate indicators of sugar we possess ; if distilled water be used as a solvent such minute traces of sugar as 1 in 120,000 can be distinctly demonstrated. The following table[1] shows the delicacy of the more common tests for sugar in aqueous solution :—

Fehling's test	= 0·0008	per cent.
Trommer's test	= 0·0025	„
Nylander's test	= 0·025	„
Fermentation test	= ·1-0·5	„
Phenyl-Hydrazin test	= 0·025-0·05	„
Polarimetric test	= 0·025-0·05	„

No normal urine, however, will give the slightest indication of the presence of sugar on the addition of many times 0·0008 per cent. of dextrose ; this at once leads to the conclusion that urine must contain something which prevents sugar, when present in small amount, giving any appreciable reaction with Fehling's solution. Pavy explained this phenomenon, on the assumption that the action of the alkaline constituents of Fehling's solution on the nitrogenous constituents of the urine, might generate free ammonia in quantity sufficient to hold the reduced cuprous oxide in solution, and so form no precipitate. In order to test this, various artificial urines were made up, each containing the chief nitrogenous constituents of urine[2] in slightly varying proportions, together with the ordinary salts ; to these artificial urines, traces of sugar from 0·0008 per cent. upwards, were added ; on boiling these with Fehling's solution, however, there was a very distinct reduction in every case, and it was found that even the uric acid present was quite sufficient to give a fairly distinct reduction without the addition of any sugar ; indeed, it was obvious from these experiments that normal urine must contain much more uric acid than is necessary to reduce Fehling's solution in ordinary aqueous solution in the absence of interfering substances. Moreover, the addition of free ammonia in quantities greater than would likely

1. Simon's *Clinical Diagnosis*, 1904, p. 522.
2. Except kreatinin.

be present in urine, either free or loosely combined, did not seem to affect the delicacy of the test to any appreciable extent. Were the reaction due to ammonia present in loose combination, boiling the urine with the alkaline part of Fehling's solution for a comparatively short time, should be sufficient to remove it; urines, however, to which small amounts of dextrose have been added previously, do not give any more indication of the presence of sugar after boiling than before.

An estimation of the amount of ammonia given off from an artificial urine, which showed very distinctly the presence of small traces of sugar with Fehling's solution, and from a normal urine which gave no indication of a relatively much greater amount of sugar, resulted in a somewhat greater yield of ammonia having been obtained from the artificial urine.

All this is contrary to the idea that ammonia is the substance that prevents small amounts of sugar from showing with Fehling's solution in normal urine; while it may have a certain effect in some cases, it is ordinarily evolved in too small an amount to markedly interfere with the reaction. As the result of many experiments, it was found that the substance normally present in urine which tends to prevent small quantities of sugar reacting with Fehling's solution in the ordinary way and interferes most with the test is kreatinin. As Allen's modification of this test does not get rid of all the kreatinin present, it is not quite trustworthy for small amounts of sugar.

Reactions of Kreatinin with Fehling's Solution and the Manner in which it Modifies the Reaction of Dextrose with the Reagent

Despite the amount that has been written in regard to Fehling's test for dextrose, I have been quite unable to find any definite statement of the exact manner in which kreatinin modifies the results of this test. From the statements made in the ordinary text books that kreatinin is an 'interfering' or 'reducing' agent, it might be inferred that a strong aqueous solution of kreatinin when heated with Fehling's

solution would give a reduction and precipitate which might be mistaken for sugar. This, however, is not so. Kreatinin does reduce Fehling's solution in the sense of decolourizing the blue solution, but a pure sample gives no precipitate unless the boiling process is very much prolonged. If a small quantity of kreatinin, such as might be present in urine, be dissolved in some water and Fehling's solution added no change whatever results, unless on prolonged boiling; the addition of larger amounts reduces the blue colour till ultimately a clear solution is obtained; this clear solution becomes yellow if more is added, but no precipitate is obtained.

In the specimens[1] used in this investigation, the following results were obtained on boiling solutions of kreatinin with an equal volume of Fehling's solution :—

1 mgr.	kreatinin in	1 c.c. H_2O	= No change whatever even on somewhat prolonged boiling; no change after cooling.
3 mgr.	„	1 c.c. „	= No change on boiling; the colour seemed to be slightly reduced on cooling, but this is hardly apparent.
5 mgr.	„	1 c.c. „	= Partially decolourized on boiling; on cooling distinct reduction of colour, but still fairly blue.
9 mgr.	„	1 c.c. „	= Distinct decolourization on boiling; after cooling showed almost clear solution.
15 mgr.	„	1 c.c. „	= Decolourized on boiling, giving yellow solution distinctly yellow on cooling; no ppt.

G. S. Johnson[2] found that 0·0284 grm. of sarcous kreatinin reduced 40 c.c. of Pavy-Fehling solution; 40 c.c. of Pavy-Fehling solution being equivalent to 4 c.c. of ordinary Fehling's solution it follows that 1 c.c. of Fehling's solution would be reduced by $\frac{0{\cdot}0284}{4}$ gram = 7·1 mgr. kreatinin. This corresponds approximately with results given above. Since the amount of kreatinin present in urine is variously estimated at an average of from ·5 to 2 grammes per diem. (= 0·33 – 1·33 mgr. per c.c. taking 1,500 c.c. as normal amount of urine per diem) it cannot decolourize the ordinary Fehling's solution to any appreciable extent

1. Samples were obtained from Merck and from Grübler; kreatinin was also prepared from the urine by Johnson's method.

2. *Proceedings Royal Society*, London, Vol. L, 1891. He put the reducing power of urinary kreatinin somewhat higher.

in virtue of its own direct reducing power even when present in excess, such as 4 grammes per diem or so; in urine, moreover, to which a sufficient amount of kreatinin has been added to give a reaction with Fehling's solution the result is somewhat different from that obtained in aqueous solution; in urine as a rule it tends to give a light greenish coloured fluid rather than a clear liquid. This direct reducing power possessed by kreatinin is therefore of comparatively little importance in regard to its interference with Fehling's test unless the kreatinin be present to a very abnormal extent.

Kreatinin, however, markedly interferes with the results of Fehling's reaction in other ways.

1. By its power of preventing small quantities of dextrose from giving a reaction with Fehling's solution.

2. By its power of modifying and masking the reaction of dextrose when present in larger amounts.

With regard to its power of masking the presence of small amounts of sugar, it is probable that the cuprous hydrate or oxide is held in solution by kreatinin, and so does not become apparent in the ordinary way. Quantities of sugar which, in aqueous solution, give quite a marked reaction with Fehling's test, give no apparent reaction whatsoever—no decolourization, etc.—after the addition of a small amount of kreatinin; if the amount of sugar present is small, a comparatively small amount of kreatinin is quite sufficient to prevent any reaction; if on the other hand, the solution contains a larger amount of sugar than can be prevented from showing by the amount of kreatinin present, a modified and ambiguous reaction results. An aqueous solution containing about ·08 per cent. of sugar, gives a very strong reaction with Fehling's test; if 1 c.c. of this solution be mixed with an equal amount of Fehling's solution, and from 2-3 mgr. kreatinin added, no change whatever is observed, even after boiling for some time; if somewhat less than 2 mgr. kreatinin be added, and the mixture boiled, no marked change is seen for some time; after cooling, however, the liquid usually undergoes a change—first it becomes greenish-blue, then greenish, and finally assumes a greenish-yellow opalescent appearance—the reaction is much modified and

changed. Stronger solutions of sugar require correspondingly larger amounts of kreatinin to prevent the reaction, but considerable quantities of sugar can be prevented from giving any precipitate by means of kreatinin, only in this case the blue solution is more or less modified in colour. Since, according to Voit, kreatinin may be present in normal urine up to about 3 mgr. per c.c., it is obvious that pathological amounts of sugar might be obscured or masked by this substance, especially in concentrated urines. Kreatinin, as stated, when present in sufficient amount to give a slight decolourization, tends to give, not a clear, but a greenish solution : this, however, is due to the modifying influence of dextrose or some other reducing substance present, seeing it never gives this in aqueous solution, and depends on the relative amounts of kreatinin and reducing substance present, which may or may not be dextrose. In these urines, however, it is often found, that subjection of the urine to yeast fermentation results in the elimination of this power of changing the blue Fehling's solution to green ; this points to traces of sugar being the cause of the change ; in fact, it would seem that in the majority of cases in which the Fehling's solution is changed to a dense green colour, dextrose, and not other reducing substances, constitutes the chief factor in the change. Repeated examination of a urine shewing this phenomenon will sometimes result in the detection of an unmistakable quantity of sugar, indicating that such urines should be regarded with suspicion. On the other hand, a urine, poor in kreatinin, and rich in some reducing substance, such as uric acid, might give the same reaction, though, for reasons mentioned below, it would probably not cause confusion. Glycuronic acid, if present, might give it however.

The power possessed by kreatinin of modifying in quite a different manner the reaction of dextrose with Fehling's solution is perhaps its most important characteristic from the clinical point of view at any rate. It is sometimes found that a urine will for some time give no indication of any decolourization or precipitate with Fehling's test, or it may give a slight greenish colouration ; this, however, may not happen till it stands for some time after heating ; soon, however, this green colour becomes more marked, till ultimately

the liquid becomes of a dirty milky green, ultimately assuming a very characteristic greenish-yellow opalescent appearance. There is generally no precipitate. This reaction is often mentioned in works on urine examination, but there seems to be no explanation of its cause or significance. Simon[1] in his book on *Clinical Diagnosis* states that not infrequently when applying Fehling's test it will be observed that upon standing when no precipitate has occurred previously the blue colour of the mixture changes to emerald green, while the solution at the same time becomes turbid, but that such a phenomenon should not be referred to the presence of sugar, as it is, in all probability, due to the action of other reducing substances. Allen,[2] on the other hand, while admitting that a urine giving a reaction similar to that described above may contain sugar, explains the phenomenon on the assumption that some of the reducing substances of urine, such as uric acid, hippuric acid, hypoxanthine, glycuronic acid, or kreatinin may be preventing the normal reactions of sugar.

The substance, however, which causes this modified action is kreatinin. The other reducing substances which may be present in the urine to a greater or less extent never seem to interfere with the ordinary sugar reaction in the manner indicated. If the amount of kreatinin present is barely sufficient to entirely prevent a reaction with Fehling's solution, there is often no result on boiling, but, soon afterwards, the greenish colouration appears, and the liquid may become gradually an opalescent, dirty, greenish fluid; if the amount of kreatinin is small, or the sugar relatively large, the reaction occurs in a short time; the reverse also holds good, and sometimes the reaction is postponed for six minutes and even longer. If to a solution acting as above a very slight amount of kreatinin be added, the reaction is postponed still longer, or may hardly be in evidence at all. The following results were obtained with solutions of dextrose in distilled water, treated with equal amounts of Fehling's solution :—

1 c.c. of ·08 per cent. sugar solution with 2½ mgr. kreatinin ...	No change even after standing for ½-hour after boiling.

1. Simon, *Loc. cit.*, p. 518.
2. Allen, *Loc. cit.*, p. 61.

1 c.c. of ·1 per cent. sugar solution with 2 mgr. kreatinin ...	No change on heating; some time after cooling, dirty yellowish opalescence.
1 c.c. of ·15 per cent. sugar solution with $3\frac{1}{2}$ mgr. kreatinin ...	Slight greenish-yellow decolourization of fluid, but no ppt. even on cooling.
1 c.c. of ·4 per cent. sugar solution with 3 mgr. kreatinin ...	Decolourized on boiling; greenish-yellow; after cooling, very distinct, dirty yellow, opalescence in fluid.

To give this modified reaction two substances are necessary:

(*a*) Kreatinin.

(*b*) Substances reducing cupric oxide, and capable of giving a precipitate in the ordinary way with Fehling's solution.

Of class *b*, the chief substances usually present in the urine are dextrose and uric acid, and in rare instances, glycuronic acid. Neither kreatinin nor any of these substances will give the reaction alone, but any one of class *b* will give it when present in certain quantities in a liquid having a certain proportion of kreatinin. Uric acid, however, will only act when present in much larger proportion than is ever likely to be encountered in urine, as its reducing power seems to be relatively small, and is easily and entirely masked by a very small amount of kreatinin. Thus, a urine containing less than the normal minimum of kreatinin would require to have a great excess of uric acid present in order to give the reaction; it is therefore probable that the reaction is seldom, if ever, dependant on uric acid. Glycuronic acid, on the other hand, does not seem to be present in appreciable quantities in urine, unless as the result of some drug which is being taken, so that dextrose is the substance most likely to give this phenomenon. In eight cases of urines giving this reaction more or less distinctly, I was enabled to demonstrate the fact, that dextrose was present to an extent beyond the usual average of 'normal' urine, and on the removal of the dextrose the reaction was not obtained; in these cases, I corroborated the fact that sugar was the substance causing the phenomenon, by a combination and modification of Crismer's Safranine test with fermentation.[1] It would thus seem that, in the majority of urines giving this modified reaction, the phenomenon ought really to be ascribed to the presence of sugar and not to the action of other

reducing substances. On the other hand, the presence of a very small amount of kreatinin might result in the production of this phenomenon with the physiological trace of sugar present in normal urines; other tests, however, demonstrate that, as a rule, it is due to a small excess of sugar. The question, however, of the significance of small amounts of sugar, while still unanswered, is yet, in many cases, of very material importance, both from a prophylactic and prognostic point of view. It is in such cases that Fehling's test is rendered obscure, and often difficult or impossible of interpretation, owing to the kreatinin present. If we take into account the number of ambiguous reactions yielded by Fehling's test in hospital practice, and generally ascribed to 'interfering' substances, but really usually due to small amounts of sugar, it is quite possible that the existence of small amounts of sugar beyond what may be considered the strict physiological limit is not so rare as is generally supposed. The constant presence of a minute percentage of sugar in normal urine has been demonstrated beyond question by many observers, and it is quite possible that occasional small amounts of sugar beyond what may be considered the normal amount may not be of much significance, more especially if observed early, and the conditions underlying its appearance modified or eliminated.

In the case of four healthy individuals whose urines were examined at short intervals—often two or three times daily—for some months, there was found undoubted evidence of small amounts of sugar in the case of two of these as indicated by Fehling's solution. This was substantiated by fermentation, and by the combination of safranine and fermentation mentioned above; in both cases it was quite transitory, and no reasons could be given for its presence.

On the Amount of Sugar which may be Added to Normal Urine Without Reacting to Fehling's Solution

This seems to vary to a considerable extent, both in regard to different individuals, and in regard to the same individual under different conditions of food. In the case of a healthy adult living on

1. The exact manner in which this was accomplished will be fully described in a later number.

milk diet, it was found that the addition of ·03 per cent. of sugar to the urine gave a greenish colourization at first, which, after some time, gave a fairly opalescent, dirty yellowish liquid ; about ·06 per cent. gave a slight precipitate with Fehling's solution. When on ordinary mixed diet the addition of ·03 per cent. of dextrose gave no change with Fehling's solution ; addition of ·06 per cent. gave a green colour on boiling, resulting in a greenish, yellowish, opalescent mixture after standing. When living almost exclusively on a meat diet the addition of ·08 per cent. of dextrose to the urine only resulted in a greenish tinge being obtained ; there was no opalescence on cooling. In the case of another urine, it was found that the addition of ·03 per cent. of dextrose gave practically no change ; ·06 per cent. gave a slight greenish-blue colour on heating, and, after cooling, an opalescent liquid. All the above only gave indications which would ordinarily be classified as due to 'interfering' substances.

Action of Kreatin

It is doubtful whether kreatin occurs in normal urine, but in alkaline urine it is alleged that it may be sometimes present. In endeavouring to get rid of kreatinin I boiled the urine for some time with the alkaline part of Fehling's solution, in order to change it into kreatin. This urine, however, acted as before, and this led to the suspicion that probably kreatin acted in the same manner as kreatinin. This view was afterwards corroborated by the use of apparently pure kreatin,[1] this substance acting towards sugar in exactly the same way as kreatinin did.

Action of Kreatinin and Kreatin in Modifying the Colour of the Precipitate Obtained with Fehling's Solution

In urines containing comparatively small amounts of sugar, but sufficient to give a distinct precipitate with Fehling's solution, it has often been noticed that the resulting precipitate tends to be yellow in colour (cuprous hydroxide) and not red as the reduced oxide. This appearance which, as observed by many investigators, is caused by

1. Samples were used in which no trace of kreatinin could be indicated by nitroprusside of sodium and alkali reaction.

kreatinin, may be obtained just as readily by the addition of a small trace of kreatin. This can be shown by adding kreatin to an aqueous solution containing sugar and treating with Fehling's solution in the ordinary way. Thus the experiment of Cipollina[1] by which a urine giving the yellow precipitate was, after long boiling with an alkali, found to give a red precipitate, probably meant that the kreatinin normally present had been destroyed by the alkali ; if it were only changed to kreatin this would in no way influence the colour of the precipitate, which would still be yellow.

Summary

Normal urine requires a very much greater amount of dextrose to give a reaction with Fehling's test, than is necessary to produce a distinct reaction in aqueous solution. The chief factor concerned in this is kreatinin, and not ammonia as suggested by Pavy. Increased amounts of sugar may be masked, within certain limits, by a corresponding increase in kreatinin, such as may be caused by change of diet.

The amount of uric acid present in normal urine is more than sufficient to react with Fehling's reagent in the absence of kreatinin. Kreatinin has a very powerful influence in preventing uric acid from giving a reaction with Fehling's test ; it is probable that uric acid is never present in large enough quantity to give a precipitate of cuprous hydrate or oxide in a urine containing even the minimum normal amount of kreatinin.

Kreatinin in the amount in which it is generally present in urine would not be likely to cause confusion in virtue of its direct reducing power.

Many of the greenish liquids obtained on heating urine with Fehling's solution are due to the influence of sugar.

The opalescent greenish milky fluid obtained in certain urines some time after boiling with Fehling's solution, depends essentially on the kreatinin present modifying the reaction of some other reducing substance ; as a rule this reducing substance is really dextrose, present in the urine in an amount greater than the average for ' normal ' urine.

Kreatin apparently acts in the same way as kreatinin.

1. *Deutsche Med. Wochenschr*, 1901.